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Comparative study of NMP preloaded and dip-loaded membranes for guided bone regeneration of rabbit cranial defects

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Abstract

Guided bone regeneration (GBR) has been utilized for several decades for the healing of cranio-maxillofacial bone defects and particular in the dental field by creating space with a barrier membrane to exclude soft tissue and encourage bone growth in the membrane protected volume. Although the first membranes were non-resorbable, a new generation of GBR membranes aims to biodegrade and provide bioactivity for better overall results. The Inion GTR™ poly(lactide-co-glycolide) (PLGA) membrane is not only resorbable, but also bioactive, since it includes N-methylpyrrolidone, which has been shown to promote bone regeneration. In this study, the effects of loading different amounts of NMP onto the membrane through chemical vapor deposition or dipping have been explored. In vitro release demonstrated that lower levels of NMP led to lower NMP concentrations and slower release based on total NMP loaded in the membrane. The dipped membrane released almost all of the NMP within 15 minutes, leading to a high NMP concentration. For the in vivo studies in rabbits, 6 mm calvarial defects were created and left untreated or covered with an ePTFE membrane or PLGA membranes dipped or preloaded with NMP. Evaluation of the bony regeneration revealed that the barrier membranes improved bony healing and that a decrease in NMP content improved the performance. Overall, we have demonstrated the potential of these PLGA membranes with a more favorable NMP release profile and the significance of exploring the effect of NMP on these PLGA membranes with regards to bone ingrowth.

1. Introduction

The concept of guided bone regeneration (GBR), which was developed into a therapy beginning in the 1980s (Nyman, Lindhe et al. 1982, Dahlin, Linde et al. 1988), is based on the formation of space to allow bone ingrowth while excluding soft tissues from entering the created compartment. The slower-growing osteoprogenitor cells can proliferate without competing with the undesired connective tissue. GBR greatly aids the formation of new bone in oral and maxillofacial tissues, which can present complications due to unusual geometries, necessity of precise application, and oral bacterial flora (Mikos, Herring et al. 2006). GBR has contributed to closing bone gaps due to loss from trauma, periodontitis, or tumor resection that are non-self-healing and require an intervention. Formerly, the only

viable option was utilizing autologous bone, which is effective, but requires harvesting bone from another site, a procedure often associated with pain and morbidity of the graft harboring site (McAllister and Haghighat 2007, Hughes, Ghuman et al. 2010).

To apply this therapy, the GBR occlusive pocket is normally achieved by the placement of a membrane to physically seal off the area from non-bony tissue. The first generation of GBR membranes consisted of expanded polytetrafluoroethylene (ePTF), a non-resorbable material (Dahlin, Linde et al. 1988, Retzepi and Donos 2010). This treatment, with the polymer's stability and non-immunogenicity, produced reliable results and has long been held as the gold standard treatment (Hammerle and Jung 2003, Retzepi and Donos 2010). However, the risk of disturbing healing with the necessary second surgery to remove the permanent ePTF membrane is a significant drawback. A second generation of resorbable membranes was developed to address this issue. Additionally, degradable membranes can potentially provide better healing as the material resorption and bone ingrowth occur simultaneously. Both synthetic polymers, including polyesters (poly(lactic acid), poly(glycolic acid), and poly(caprolactone) and their copolymers) (Milella, Barra et al. 2001, Geurs, Korostoff et al. 2008, Sculean, Nikolidakis et al. 2008, Gentile, Chiono et al. 2011) and poly(ethylene glycol) (Wechsler, Fehr et al. 2008, Ramel, Wismeijer et al. 2012), and biologically-derived materials, namely collagen (Colangelo, Piattelli et al. 1993, Parodi, Carusi et al. 1998, Wang and Carroll 2001, Tal, Kozlovsky et al. 2008, Coic, Placet et al. 2010), are the most common GBR membranes.

Although these bioresorbable GBR membranes have shown some success and obviate the need for a second surgery, on their own they generally do not actively accelerate bone regeneration. As a third generation of GBR membranes, researchers have strived to develop biodegradable, occlusive materials that are also bioactive. The Inion GTR™ membrane is composed of biodegradable co-polymers of polylactic acid (PLA), polyglycolic acid (PGA) and trimethylene carbonate (TMC) and is temporarily softened with N-methyl pyrrolidone (NMP), enabling easy handling and placement (Pirhonen, Pohjonen et al. 2006). Moreover, our lab previously discovered that NMP is a substance capable of accelerating bone formation by enhancing the effect of bone morphogenetic protein-2 (BMP-2) on osteoblasts (Miguel, Ghayor et al. 2009) and inhibiting bone resorption by

suppressing osteoclast maturation and activity (Ghayor, Correro et al. 2011). Consequently, NMP must be considered not only as a plasticizer, but also as a drug, rendering the NMP-softened PLGA-based membrane the first GBR membrane that is occlusive, bioresorbable, and bioactive.

The NMP treated PLGA membrane obtains its bioactivity through uncomplicated means, without the use of growth factors or other bioactive molecules that must maintain a proper protein structure to be effective. This simplicity can lead to a more reliable GBR membrane. Moreover, NMP is FDA-approved as an excipient in pharmaceutical formulations, inexpensive, and readily available. Its timed and sustained delivery, however, is difficult due to the small size of the molecule and its amphiphilic character. In order to optimize the bioactivity of NMP, the concentration and release profile of NMP that is optimal for bone regeneration has to be defined. For that purpose, three different concentrations of NMP were preloaded into the PLGA membrane with chemical vapor deposition and compared to the membrane dipped into liquid NMP and the gold standard of a GBR membrane, the ePTF membrane produced by Gore, in in vitro release studies and in a rabbit cranial defect model. This study demonstrates the significance of varying the NMP loading in PLGA membranes on NMP release and in vivo bone regeneration.

2. Materials and Methods

2.1 Membranes: Inion GTR™ (Inion Oy, Tampere, Finland) named PLGA 50 and GoreTex ePTFE™ (Gore, Flagstaff, Arizona, USA) named ePTFE were used in these studies.

2.2 NMP Loading of Inion Membranes: NMP was loaded onto the Inion GTR membrane with chemical vapor deposition to obtain 10, 15, and 20% NMP in the membrane, henceforth designated as PLGA 10, PLGA 15, and PLGA 20, respectively. Membranes were placed on top of a piece of filter over a petri dish with NMP in a desiccator. Deposition took place at room temperature and with the aid of a pump connected to the desiccator over a period of 1-2 days. Membranes were weighed before and after deposition to determine the percent loading. The 50% NMP loading was obtained by dipping the membrane in NMP for 20 seconds followed by air-drying for 15 minutes; these

membranes are henceforth designated PLGA 50. The dipping was performed according to the recommendation of the manufacturer.

2.3 NMP In Vitro Release: 20 mm x 15 mm rectangular membranes were placed in 50 mL of phosphate buffered saline, pH 7.4. Samples were held at 37°C and shook at 100 rpm. 200 µL was removed at each timepoint **up to 16 weeks** and stored at 4°C for later analysis. NMP concentration was measured at 220 nm using 96 well plates coated to enable reading at UV wavelengths (Corning, Corning, NY, USA). Values were compared to a standard curve of known NMP concentrations. The concentration of released NMP was equated to the equivalent membrane area (two 7 mm diameter membranes) and defect volume (100 µL) used in the in vivo rabbit experiments.

2.4 Structural analysis. The structural analysis was performed both times for four test specimens derived from the same Inion GTR membrane by cutting it in 4 pieces; 1) untreated, 2) vapor loaded with 20% (w/w) NMP, 3) vapor loaded with 20% (w/w) NMP exposed to 50 mL of phosphate buffered saline, pH 7.4 for 1 h, 4) dip loaded membrane exposed to 50 mL of phosphate buffered saline, pH 7.4 for 1h. Prior to SEM analysis the samples exposed to phosphate buffered saline had to be dried overnight by lyophilization (Christ Alpha 2-4). Next, all membranes were fixed on metallic SEM holders with fast glue. Prior to SEM analysis, membranes were sputtered with Gold (SCD 030, Baltec, Balzers Lichtenstein). Parameters for sputtering leading to a gold layer of 10 nm on the samples were as follows. Distance of sample to gold target: 60mm, sputtering time: 90 seconds, and current: 45mA. Membranes were analyzed using a Zeiss Supra V50 scanning electron microscope (SEM) (Carl Zeiss, Oberkochen, Germany) at an acceleration voltage of 5kV.

2.5 Animal model: The ethical approval of the local authorities (Veterinäramt Zurich, Switzerland) was obtained before the start of the experiments. 15 new Zealand white rabbits were sedated by ketamin and further anesthetized by a Halothan-N₂O inhalation method. The surgical area was clipped and prepared with iodine for aseptic surgery. A linear incision was made from the nasal bone to the midsagittal crest. The soft tissues were reflected and the periosteum was dissected from the site (occipital, frontal, and parietal bones). Four 6 mm craniotomy defects were created (two in the parietal and two in the frontal bone) with a 6 mm trephine in a dental hand piece. The surgical area was flushed

with saline to remove bone debris. To avoid any dural perforation the defects marked half way through the bone by the trephine were finally made by a round burr creating a 6 mm defect. Since the defect size was exactly 6 mm membranes on the dural side were chosen to be 7 mm in diameter and fixed by the pressure from the dura. Another rectangular membrane of 10x10mm was placed above the defect. This experimental scenario with membranes on both sides of the cranium has been developed by Linde and coworkers (Dahlin, Alberius et al. 1991). To determine the effect of released NMP on an empty defect, each defect in the parietal bone treated with a membrane was paired with a corresponding empty defect in the other parietal bone. The same was done for the frontal bone. The pairs were assigned randomly to the animals. To stabilize their positioning the two membranes of the top were sutured together and the entire unit sutured to the remaining periost. The soft tissues were closed with sutures. After the operation, analgesia was provided by injection of Novalgin (50 mg/kg). Four weeks after operation the rabbits were sacrificed after sedation with barbiturates by an overdose of ketamin and the calvarial bone was excised.

2.6 Radiography and histology. Specimens were radiographed using a dental radiography unit with ultra-speed dental films (Eastman Kodak Company, NY, USA). The radiographs were photographed, scanned and later used to localize the middle section of the defects. After radiography, the samples were first prepared with a sequential water substitution process that involved 48 h in 40% ethanol, 72 h in 70% ethanol (changed every 24 h), 72 h in 96% ethanol and finally 72 h in 100% ethanol. Samples were placed in xylene for 72 h for defatting the recovered bone (changed every 24 h). Next, infiltration was performed by placing the samples in methyl methacrylate (MMA) for 72 h (Fluka 64200) followed by three days in 100 ml MMA + 2 g di-benzoylperoxide (Fluka 38581, dehydrated in desiccator) at 4 °C. Samples were embedded by placing them in 100 ml MMA + 3 g di-benzoylperoxide + 10 ml plastoid N or dibutylphtalate (Merck 800 19.25) and allowing polymerization to occur at 37 °C in an airtight water bath. 4.5 µm sections were prepared from the middle of the defects and stained with Goldner Trichrome. Digital images were taken and processed with an image analysis program (Adobe Photoshop).

2.7 Analysis of data: The digital image of the vertical section from the middle of the defect was taken with a scale (mm) in order to localize the defect margins, which were six mm apart. In combination with the calvarial bone thickness of the surrounding bone, the total area of the defect could be reconstructed. Using the Adobe Photoshop Elements program, the total pixel number of the defect area could be determined. The part of the total defect filled by bone was also determined and expressed by pixel number of the bony area. Percentage of bone healing was calculated as: Bony regeneration [%] = pixel number of the bone area X 100/total pixel number of defect.

Defect bridging was determined in the middle section to compare the extent of bone coverage across the x-axis of the defect. First, the areas with bone tissue were projected onto the x-axis. Next, the stretches of the x-axis where bone formation had occurred at any level were summed up as described earlier (Kruse, Jung et al. 2011). Bone bridging is given in percentage of the defect width (6 mm) where bone formation has occurred.

2.8 Statistical analysis: Mean values and standard deviations were calculated for the percentage of bone regeneration and bone bridging. The significance of differences was evaluated by the Kruskal-Wallis test followed by the Mann-Whitney test. The significance of differences between membrane treated and the corresponding untreated defect was determined by the Wilcoxon signed rank test. The limit for significance was set to <0.05. Statistical analysis was performed by using a statistical software package (IBM SPSS Statistics Version 21 for Windows).

3. Results

3.1 In Vitro NMP Release

PLGA membranes either preloaded with NMP using chemical vapor deposition or dipped into liquid NMP were assessed for their in vitro release of NMP. As shown in Figure 1A, the method of dipping the PLGA membrane into NMP and then letting it dry, which results

in approximately 50% loading, leads to almost immediate loss of all of the NMP. After four minutes, 81.7% was released and over 95% was released after just 15 minutes. Moreover, the concentration in the first hour of the equivalent membrane and defect size as used in the in vivo experiments reached 130 mM (Figure 1C). In contrast, the preloaded membranes released NMP at a much slower rate. The PLGA 10 and PLGA 15 have similar curves, releasing about 20% after 7 hours and 37% after 24 hours (Figure 1A). The membranes with 20% NMP released significantly more than the other two preloaded conditions, beginning at 90 minutes. After 7 and 24 hours, PLGA 20 released almost 57% and 73% of the NMP, respectively. In the time intervals 0-1 hour, 1-4 hours, 4-9 hours, and 9-24 hours, the PLGA 10 and PLGA 15 never reached an equivalent concentration of more than 5 mM (Figure 1B). However, the PLGA 20 released NMP to accumulate a concentration of at least 8.7 mM and up to 14.4 mM during these time intervals. The release from the PLGA 20 samples was prolonged over 16 weeks. The NMP release has already started to level out after one day and has almost reached a plateau by one week with 83% released. Over the course of the 16 weeks, additional NMP is continually slowly deposited in the supernatant, with a cumulative release of 96.6% after 16 weeks. The amount of loading has a pronounced effect on the release curve and local concentration of NMP.

3.2 Structure of the membranes

The type of loading the membrane with NMP influences the structure of the membrane. SEM analysis revealed that vapor deposition to 20% (w/w) NMP (PLGA 20) increases the thickness of the membrane in average from 236 μm to 266 μm (Figure 2A, 2B). Upon exposure to phosphate buffered saline for 1h and lyophilization the membrane which underwent NMP loading via vapor deposition (PLGA 20) was in average 249 μm thick, the dip loaded membrane (PLGA 50) in average 298 μm (Figure 2C, 2D). This marked increase in thickness of the dip loaded membrane in comparison to the vapor loaded membrane after exposure to phosphate buffered saline and the release of the majority of NMP is due to an increased porosity of the outer layers of the dip loaded membrane (Figure 2C, 2D lower panel) which has been described earlier (Pirhonen, Pohjonen et al. 2006).

3.3 Rabbit in Vivo Studies

During the operation of the rabbits, it was evident that the ease of handling of PLGA membranes decreased with the NMP amount. With lower amounts of NMP, the membrane was stiffer, interfering with ease of membrane placement. After operation, no adverse reaction to any of the membranes occurred. All the animals remained in good health.

In Figure 3, examples of some histological sections from the middle of the defect are shown. Although not shown, the PLGA 50 histology appears similar to the other PLGA membranes. More bone in-growth occurred with the PLGA membranes than the ePTFE. The bone formed during the 4-week repair phase is more of a cancellous type of bone rather than cortical bone. A cellular interaction with the membrane was not observed nor was bone deposited onto the membrane. A fibrous capsule was detected surrounding all of the membranes (including PLGA 20, not shown to maximize size of the images), with its thickness ranging from approximately 10 to 100 μm (Figure 4). Capsule thickness and NMP content did not appear to correlate either positively or negatively.

Evaluation of the level of bony regeneration of the defect revealed that the use of membranes (GBR) improved bony healing and that a decrease in NMP content improved the performance of the respective membrane (Figure 5). All of the defects treated with membranes showed significantly more bone growth than their corresponding untreated controls except the PLGA 50. Both the PLGA 10 and PLGA 15 demonstrated significantly higher bony regeneration, both with an average of approximately 65%, than the ePTFE membrane. The determination of bone repair via pixel number is focused on the area with bone structures and, as is illustrated in Figures 3 and 4, the structure of the newly formed bone is more cancellous and encloses a lot of area not filled with solid bone. The results in empty defect controls located adjacent to NMP releasing membranes indicate that NMP spill over did not occur or alone without the GBR membrane did not improve bone regeneration.

The defect bridging, which evaluates the bone projected onto the x-axis, also was significantly increased for the defects treated with a membrane compared to the untreated defects, excluding the PLGA 10 and ePTFE (Figure 6). The PLGA 10 facilitated significantly greater bone bridging than the ePTFE membrane and the other preloaded PLGA membranes indicate a higher level of defect bridging, although not significantly.

Similar to the bony regeneration data, the empty defect controls did not show an effect of NMP alone on defect bridging.

4. Discussion

Calvarial models are the most selective models for bone regeneration because the poor blood supply and the membranous (cortical) structure of the bone have an adverse effect on bone healing (Schmitz and Hollinger 1986). In this project, no attempt was made to produce a critical size defect that by definition would not heal during the lifetime of an animal and its healing would require osteoinductive agents, like bone morphogenetic proteins (BMPs). Therefore, four single 6 mm in diameter defects instead of one 15 mm in diameter critical size defect per animal were created. The latter one would put the focus on the mechanical properties of the membrane needed to stretch the 15 mm or would demand for the use of bone substitute materials creating additional variables in this model. The size of the defect with 6 mm was selected to resemble the typical magnitude of an osteotomy gap in maxillofacial surgery and defects in dentistry. This strategy provides the possibility to evaluate the interaction between spontaneous bone regeneration and the influence of different GBR membranes on the healing response, including the influence of release profiles of bioactive substances from membranes.

The overall results of this rabbit cranial defect study demonstrate that GBR is very effective in promoting the bony healing of such defects. Although ePTFE is non-degradable, this membrane was used as a control because it is the original gold standard membrane for guided bone regeneration, its reliability, synthetic nature, and purity. The use of a degradable collagen based membrane as control would have shifted the focus of our study on NMP release to differences in mechanical properties and would demand for the use of a bone substitute material. The clinical drawbacks from needing to remove the ePTFE membrane are inconsequential in this study due to the time frame of the experiment. Compared to the gold standard ePTFE from Gore, all PLGA-based membranes performed at least equally well, if not better, depending on the analysis type. Moreover, in a clinical setting, the non-degradable ePTFE membrane necessitates an

additional surgery to remove it, while the PLGA membranes will resorb. Although the PLGA membranes overall result in greater bone formation, it is evident that the NMP loading on the PLGA membrane influences its performance. The PLGA 10 and 15 demonstrated significantly more bony regeneration than the ePTFE (Figure 5). Additionally, the PLGA 10 achieved significantly better defect bridging (Figure 6). From these results, it appears that the lower NMP loading linked with a slower NMP release produces greater bone regeneration. Although the lower NMP loading leads to greater regeneration, it is not entirely possible to determine if the loading method and the respective NMP release profile or the amount of NMP leads to differences.

As clearly demonstrated with the in vitro results (Figure 1), the NMP release profile from the PLGA membranes varies based on the loaded amount and the loading methodology: dipping or vapor deposition. The vapor deposited membranes retain NMP to a much greater extent than the dip-loaded membrane. As demonstrated with the long term data of PLGA 20, a majority of the NMP is released over the course of a week whereas the dip-loaded membrane loses all NMP within minutes. This result is most likely due to the fact that the majority of NMP remains in the outer layer of the membrane with dipping, since NMP is only allowed 15 minutes to diffuse into the PLGA. Vapor deposition, however, allows the NMP time to diffuse throughout the membrane, since loading occurs over a time period of 1-2 days. The PLGA 20, which has the highest amount of NMP loaded with chemical vapor deposition, releases a significantly higher percent of NMP than the PLGA 10 and 15 as well as has a higher NMP concentration at every interval, although not always significantly. The fast burst release of NMP from the dipped PLGA membrane, PLGA 50, leads to an extremely high concentration of 130 mM in the first hour, which is potentially toxic to cells. Our previous experience has indicated that C2C12 cells are not viable with NMP concentrations greater than 10 mM (data not shown). The vapor deposited membranes, PLGA 10, PLGA 15, and PLGA 20; all maintained NMP concentrations lower than 10 mM at all calculated time intervals except 1-4 hours for PLGA 20. Although the in vivo situation is greatly complicated, these data illustrate the possibility of damagingly high NMP levels with the PLGA 50. Despite the fact that NMP is safe and approved in humans as an excipient by the FDA, it can be locally toxic at high levels (Davies, Ajami et al. 2013, Pelaez-Vargas, Gallego-Perez et al. 2013). Moreover, a longer release period, as in the case with the vapor deposited membranes and particularly

PLGA 10 and 15, may promote NMP's BMP-enhancing effects over an extended time period, possibly inducing greater bone growth. It is possible that both of these factors contributed to the improvement with lower NMP loading. The mechanical properties of the NMP membrane also change with the loading and release of NMP, which could contribute to differences in bone regeneration. However, the 6 mm cranial defect model is not sensitive to differences in the mechanical properties, as long as they are sufficient stiff so that a properly fixed membrane covers the defect without collapsing. Therefore we believe that differences in bone regeneration are mainly due to differences in the release kinetic of NMP which was shown in previous work to have bone enhancing effects (Miguel, Ghayor et al. 2009).

Although the membranes with the lowest amount of NMP performed best, there are practical considerations that are important in a clinical setting that could favor other membrane formulations. From the handling characteristics, only the preloaded PLGA 20 is suitable for GBR in dentistry. When the NMP content is below 20%, the membrane is too stiff for this application. As mentioned previously, reducing NMP content below 20% led to better results. In order to develop a second generation of the PLGA membrane, we have to balance the NMP effect as a plasticizer and the pharmaceutical effect of NMP, which is lethal to cells at higher concentrations.

Another aspect that should not be overlooked is that with the use of a preloaded membrane we lose the capability of tissue integration (Fig. 4). As previously demonstrated, the PLGA membranes that are dipped into NMP integrate with the tissue, with extensive ingrowth of cells and osteoid and new bone formation adjacent to the membrane (Pirhonen, Pohjonen et al. 2006). It is suspected that the surface roughness plays a crucial role in tissue integration (Figure 2). Before being placed in NMP, the polymer surface is dense with a smooth surface, but it obtains a three-part structure with a dense inner layer and outer rough porous layers upon dipping in NMP (Pirhonen, Pohjonen et al. 2006). It was previously shown that this smooth PLA/PGA/TMC film does not allow tissue integration into the membrane, while the roughened surface does promote incorporation. Simion et al. have shown that titanium-coated ePTFE membranes with high surface roughness integrated into the host tissue to a much greater extent in a GBR model (Simion, Dahlin et al. 1999). Additionally, other studies have described the positive relationship between surface roughness and osseointegration (Hayes and Richards 2010,

Choong, Yuan et al. 2012, Davies, Ajami et al. 2013, Pelaez-Vargas, Gallego-Perez et al. 2013). This topography mimics the natural bone remodeling situation where active osteoclasts create carved out pits and lacunae. Therefore, the membrane surface needs to be roughened prior to loading with NMP. If the roughening leads to an increased flexibility of the membrane, the NMP content could also be reduced and optimized for its pharmaceutical action. Moreover, since the PLGA membranes are resorbable, long-term cell integration of the membrane may not be important for the clinical outcome.

Another cellular response that may not have long term consequences is the formation of a fibrous capsule that was found with all of the membranes (Figure 4). Since the PLGA membranes degrade, the fibrous capsule may break down once the material is no longer present, imparting no extended negative effects.

5. Conclusion

PLGA membranes loaded with various amounts of NMP all demonstrated bone regeneration that is equal to or better than the gold standard ePTFE membrane in a rabbit calvarial defect model. By utilizing vapor deposition for loading, we developed a slow NMP release system from PLGA. Differences between the PLGA membranes with different NMP loading were detected in the in vivo bone regeneration as well as in vitro NMP release profiles. The release curve of NMP may influence its performance. These results indicate the importance of analyzing and optimizing NMP release. Overall, we have demonstrated the potential of these PLGA membranes and the significance of exploring the effect of NMP on these PLGA membranes with regards to bone ingrowth.

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Figure Captions

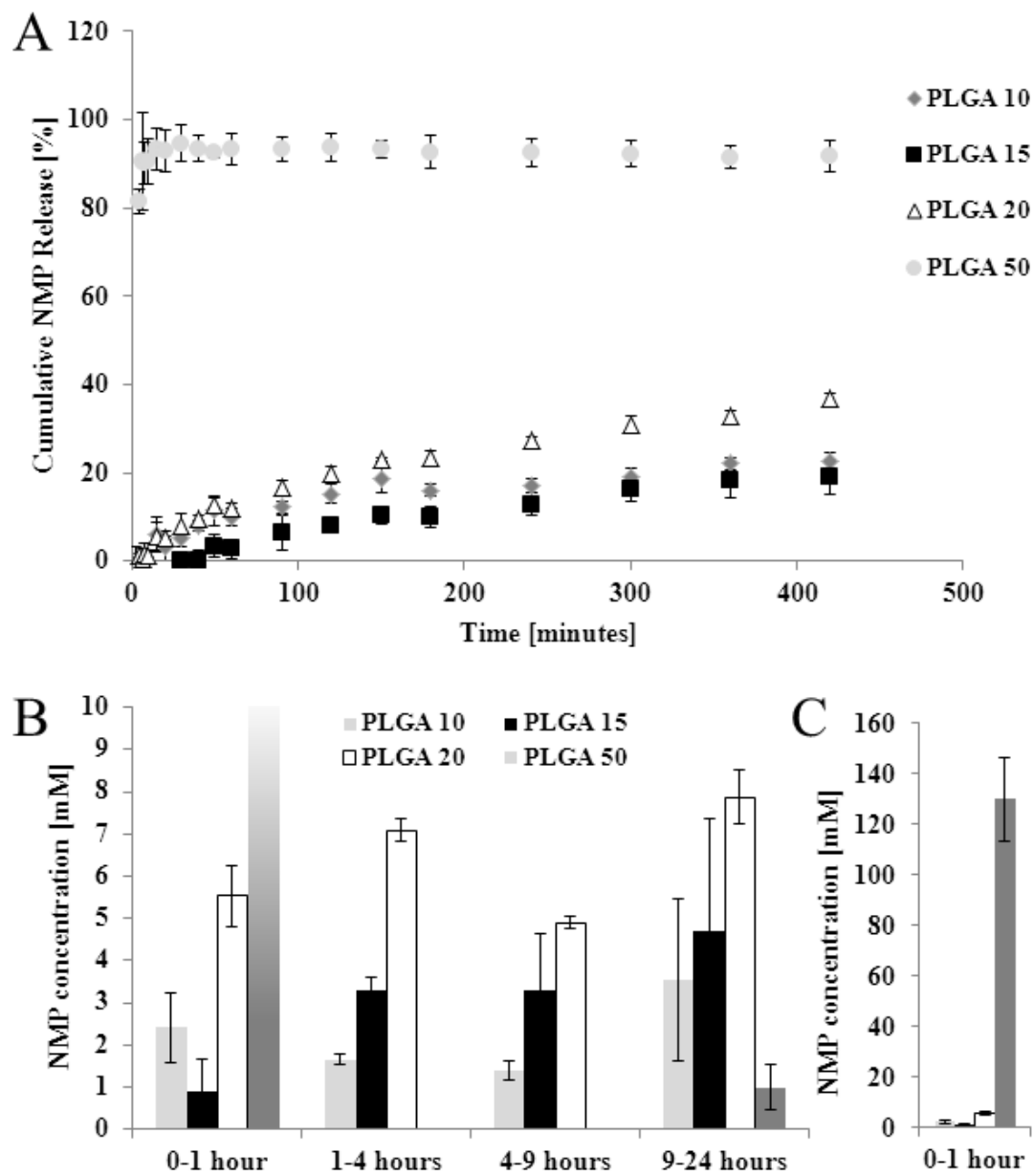


Figure 1: In Vitro NMP Release. A) Cumulative percent release of NMP from PLGA 10, PLGA 15, PLGA 20, and PLGA 50 membranes over 7 hours. B) Calculated NMP concentration in the calvarial bone defect between 0-1 hour, 1-4 hours, 4-9 hours, and 9-24 hours for PLGA 10, PLGA 15, PLGA 20, and PLGA 50. Fading indicates that the PLGA 50 data point at 0-1 hour extends past the y-axis. C) Calculated NMP concentration in the calvarial bone defect between 0-1 hour for PLGA 10, PLGA 15, PLGA 20, and PLGA 50

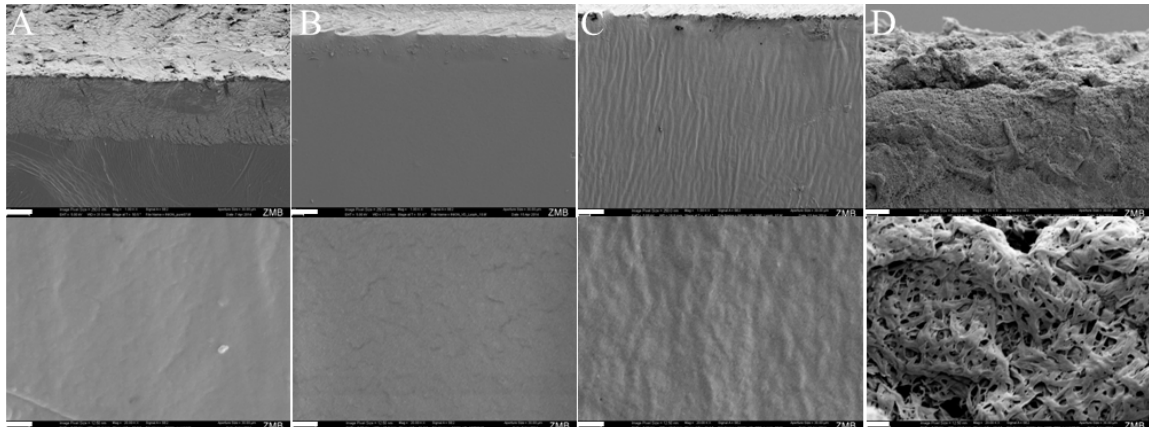


Figure 2: Scanning electron microscope images of the Inion GTR membrane at lower and higher magnification. A) Inion GTR membrane, B) PLGA 20; C) PLGA 20 (vapor deposition) exposed to phosphate buffered saline and lyophilized, D) PLGA 50 (dip loading) exposed to phosphate buffered saline and lyophilized. In the upper panel the white bars represent 20 µm; in the lower panel 1 µm.

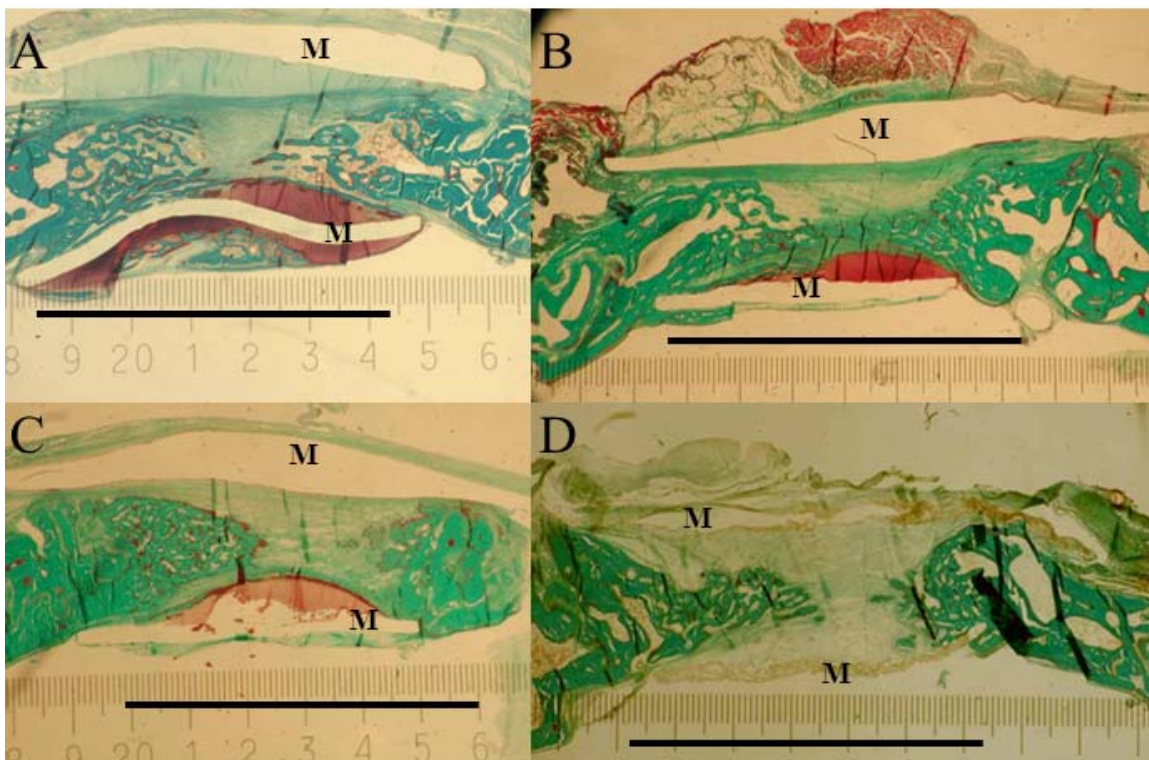


Figure 3: Trichrome stained histological sections of defects created in rabbit calvarial bone. M indicates the membrane and the solid black line marks the length of the defect. Trichrome staining stains bone green and osteoid red. No encapsulation of the membrane was seen. Ingrowth of cells did not occur. A) PLGA 10 B) PLGA 15 C) PLGA 20 D) ePTFE

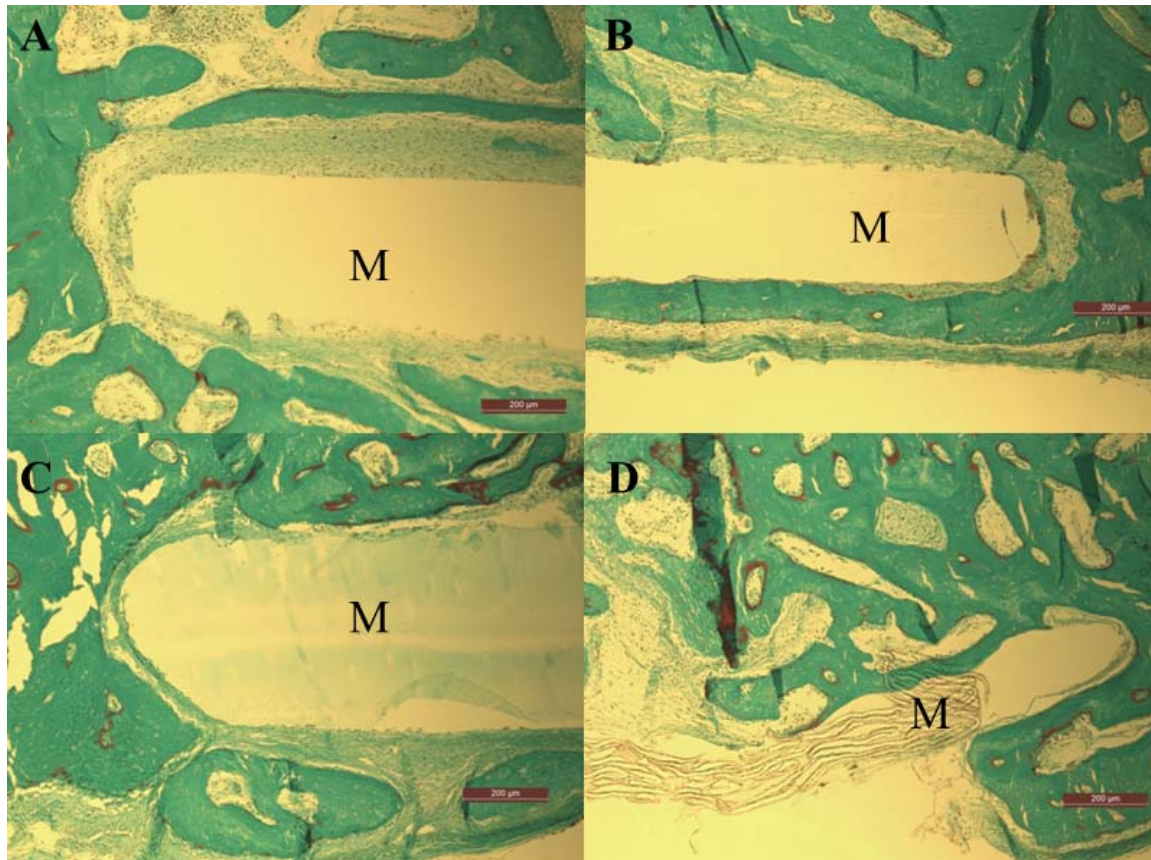


Figure 4: Interaction between different membranes and bone. A) PLGA 10 B) PLGA 15 C) PLGA 50 D) ePTFE. M indicates the membrane. Trichrome staining stains bone green and osteoid red. All membranes were surrounded by fibrous tissue of a thickness between 10 μm and 100 μm. Ingrowth of cells or a direct deposition of bone onto the membranes did not occur. The red bar indicates 200 μm.

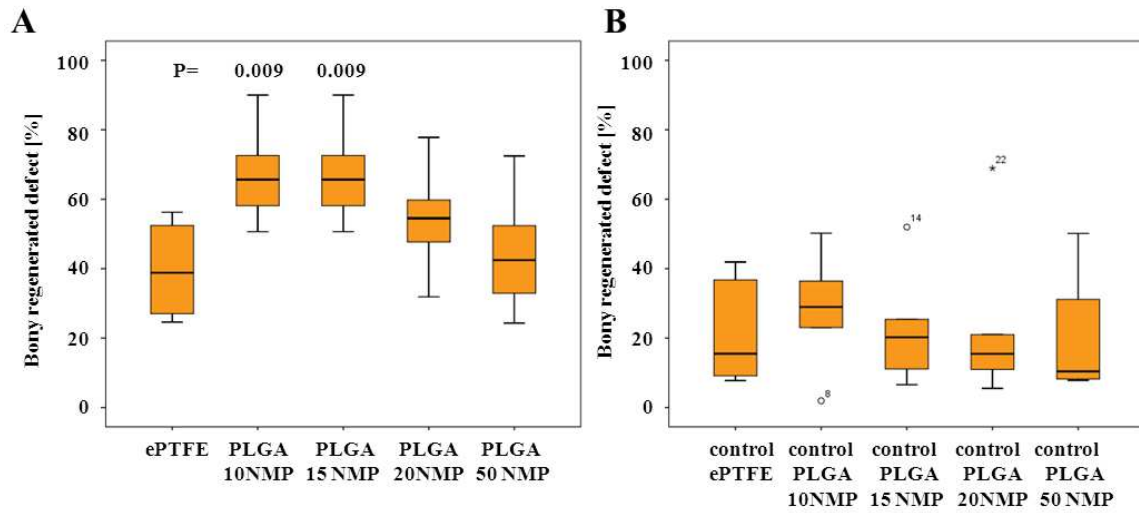


Figure 5: Bony regeneration of the defect. The bony regeneration of the defect was determined based on percentage of pixel number A) Defects treated with membranes and B) the corresponding untreated defects. Compared to the ePTFE membrane, bone regeneration was significantly increased in defects treated with PLGA 10 or 15 NMP. P values are provided. The difference between membrane treated and corresponding empty defect was significant for all conditions except the PLGA 50 pairs.

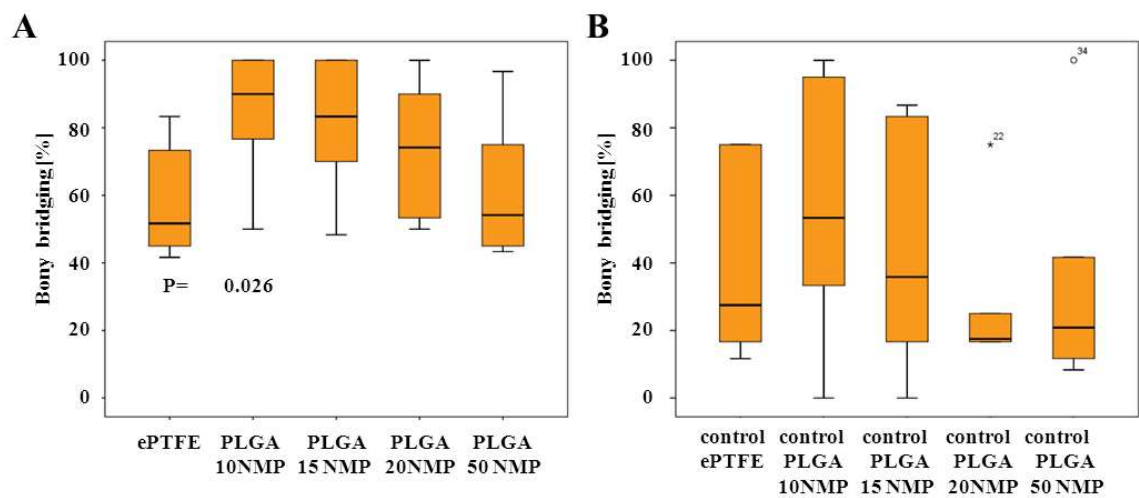


Figure 6: Defect bridging. Defect bridging as the percentage of the 6 mm defect bridged by bone in A) samples treated with membranes and B) the corresponding empty defects. Compared to the ePTFE membrane, bone bridging was significantly increased in defects treated with PLGA 10. P value is provided. The difference between membrane treated and corresponding empty defect was significant for PLGA 15, 20 and 50.